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Award Number: W81XWH-10-1-0749

TITLE: BRMS1 Suppresses Breast Cancer Metastasis to Bone via Its Regulation of MicroRNA-125b and Downstream Attenuation of TNF-Alpha and HER2 Signaling Pathways

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REPORT DATE: October 2011

TYPE OF REPORT: Revised Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| October 2011 | Revised Annual Summary | 30 September 2010 – 29 September 2011 |

**4. TITLE AND SUBTITLE**

BRMS1 Suppresses Breast Cancer Metastasis to Bone via Its Regulation of MicroRNA-125b and Downstream Attenuation of TNF-Alpha and HER2 Signaling Pathways

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-10-1-0749

**5c. PROGRAM ELEMENT NUMBER**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

University of Texas MD Anderson Cancer Center
Houston, TX 77030

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
BRMS1 is a metastasis suppressor that affects several steps of the metastatic cascade and potently inhibits metastases of many cancer types to various secondary sites. Here we show that BRMS1 has only a marginal effect on expression of HER2 and microRNA-125b. We also failed to detect TNF-alpha in cell lines utilized in this study by several methods. However, we confirmed a strong inverse correlation between expression of HER2 and BRMS1 in human breast cancer specimens. Moreover, we confirmed that cells stably expressing miR-125b exhibit reduced levels of HER2, as compared to vector only cells. In addition, our studies aimed at examining BRMS1 effect on anoikis demonstrate that BRMS1 expressing cells are inherently unable to respond to microenvironmental changes, as they display a significant delay in adhesion to matrix. Mechanistically, these cells in a time-dependent manner exhibit reduced activation of integrins and signaling molecules responsible for focal adhesion/cytoskeleton coupling. Lastly, we have preliminary evidence that BRMS1-mediated adhesion changes may be due to a time-dependent inhibition of mesenchymal-to-epithelial transition, which may also play a role in BRMS1 ability to suppress metastatic dissemination. In conclusion, these findings may explain why BRMS1-expressing cells show a greatly decreased survival in circulation in vivo and enhanced anoikis in vitro.

**15. SUBJECT TERMS**
BRMS1, metastasis, anoikis, adhesion

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
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## 1. Introduction

Our preliminary results suggested that human metastatic breast cancer cells with restored BRMS1 expression significantly downregulated their TNF-α and HER2 expression. Moreover, based on our recently published microRNA array data, we identified miR-125b as one of the microRNAs increased by BRMS1 expression (1). MiR-125b is one of the most consistently deregulated miRs in breast cancer and, similar to BRMS1, was shown to be significantly downregulated in HER2+ breast cancers (2). Consequent analysis of the 3' UTR region of HER2 revealed target sites for miR-125b, while its overexpression in HER2-dependent breast cancer cell lines showed decreased cell mobility and invasiveness (3-4). Additionally, mir-125b targets TNF-α directly for post-transcriptional repression (5), suggesting that this miR is, at least partially, responsible for regulation of HER2 and TNF-α in breast cancer. Therefore, I hypothesize that BRMS1 regulates TNF-α and HER2 expression via its regulation of miR-125b.

## 2. Results

PART I.

Before implementing labor- and time-intensive work outlined in the original grant proposal, we first set off to confirm our original preliminary data that served as the basis for our hypothesis. Below is the summary of our findings.

### 2.1. Expression of BRMS1 is inversely correlated with expression of HER2 in human breast cancer biopsy samples

To confirm our previously published results (6), we examined BRMS1 expression in primary tumor biopsies of 24 breast cancer patients with known HER2 status (12 patients clinically diagnosed with HER2+ and 12 patients – with HER2- disease). As shown in Figure 1 and Table 1, there is a strong inverse correlation ($p=0.013$) between histologically scored expression of HER2 and BRMS1, supporting our hypothesis that BRMS1 may negatively regulate HER2 expression.

To obtain data shown in Table 1, semi-quantitative analysis of immunoreactivity was ranked into three groups according to the percentages of positive tumor cells: negative and low positive (-/+, 0-25%), medium positive (++, 26-50%), and high positive (+++, >50%). Immunoreactivity was scored based on a well-established immunoreactivity score system in which immunoreactivity score was generated by incorporating both the percentage of positive tumor cells and the intensity of staining (7). Staining intensity was based on cross-product of the percentages of tumor cells staining at each staining intensities (H score). Immunoreactivity was assigned as described previously based on at least 200 cells counted (8). Slides were read by a blinded pathologist. Data were analyzed using Pearson Chi-Square test.

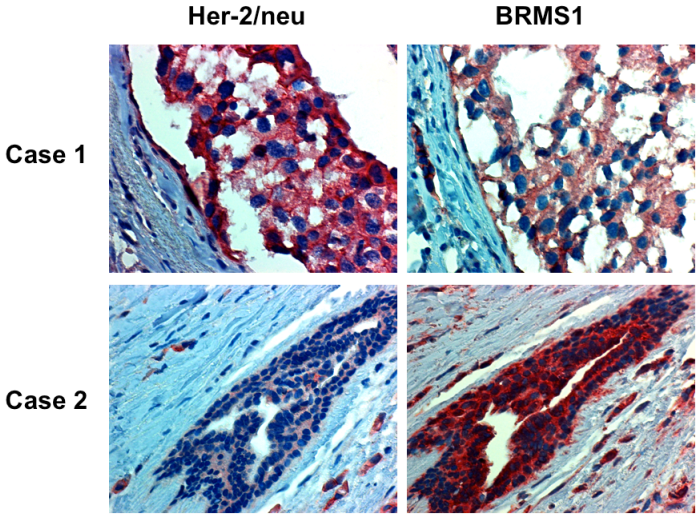


**Figure 1. Expression of BRMS1 is inversely correlated with expression of HER2 in human breast cancer biopsy samples.** Twenty-four biopsy samples from breast cancer patients diagnosed with HER+ or HER- disease were analyzed for expression of HER2 and BRMS1 by immunohistochemistry performed on consecutively cut sections. Histologic staining was analyzed by a blinded pathologist who enumerated corresponding staining. Corresponding data are shown in Table 1.

**Table 1.**

| | | BRMS1 expression | | | *p=0.013* |
|---|---|---|---|---|---|
| HER2 expression | | -/+ | ++ | +++ | Total number (% of total) |
| | -/+ | 0 (0%) | 2 (8.3%) | 6 (25%) | 8 (33.3%) |
| | ++ | 1 (4.2%) | 0 (0%) | 5 (20.8%) | 6 (25%) |
| | +++ | 5 (20.8%) | 4 (16.7%) | 1 (4.2%) | 10 (41.7%) |
| | Total number (% of total) | 6 (25%) | 6 (25%) | 12 (50%) | 24 (100%) |

These experiments were outlined in Task 3.1a (Aim 3, months 24-30). While the sample size we used was small (we originally proposed a 170 patient tissue array, while only 24 patient samples were used), we wanted to confirm that our homemade antibody raised against BRMS1 (developed by Dr. Welch's lab) produced reproducible results, since human tissues are always of a limited supply. In the process of performing these verification studies, we discovered that BRMS1 antibody produces very high background, making it difficult for our staff pathologist to conclusively distinguish positive and negative cases.

## 2.2. MiR-125b decreases HER2 expression level, but has no effect on cell proliferation

We then confirmed that miR-125b targets HER2, reducing its protein level. For these studies, we requested miR-125b construct previously described in (3) and established stable cell lines using parental MDA-231 and MDA-435 breast cancer cells (Figure 2A). As expected, in both cell lines expression of miR-125b resulted in lower expression of HER2 (Figure 2B). Surprisingly, as shown in Figure 2C we could not reproduce the previously published observation that expression of miR-125b suppresses cell proliferation (4). One possible explanation for this phenomenon is that levels of miR-125b expression obtained in our cells were insufficiently high to inhibit cell proliferation.

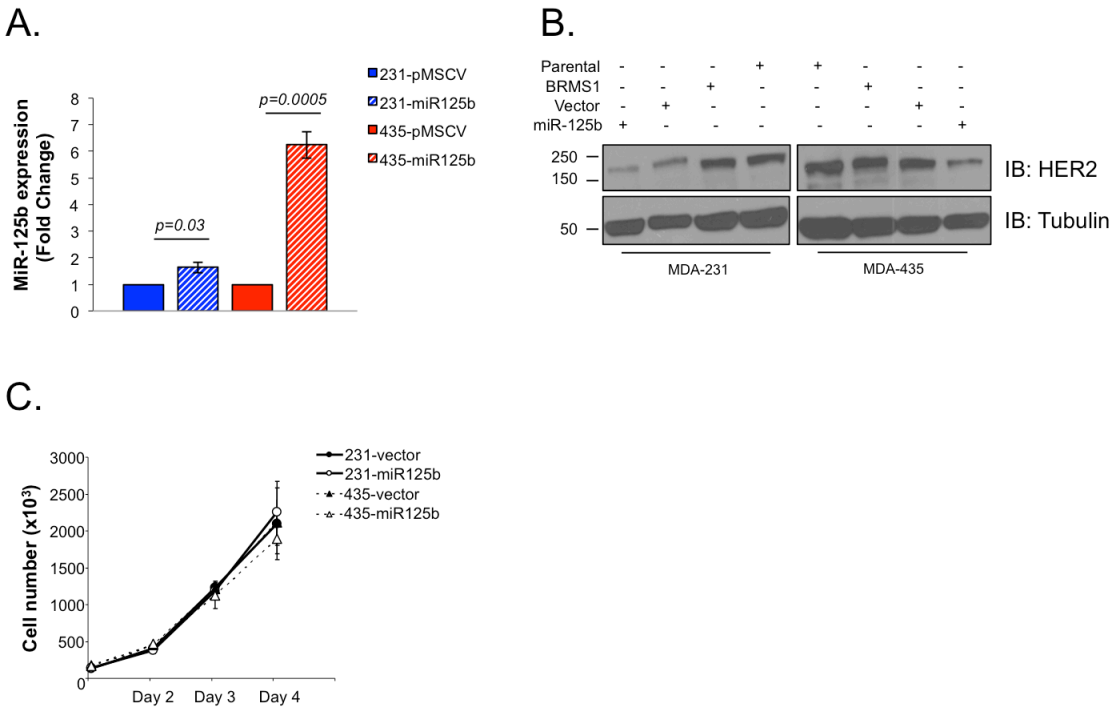


**Figure 2. MiR-125b decreases HER2 expression. A**. Levels of miR-125b were assed by qPCR in cells stably expressing miR-125b contract. Data shown are relative miR-125b expression

normalized to vector control cells (designated pMSCV). Data are representative of cells plated in triplicate and shown as mean ± SEM. Statistical significance was assessed by T-test. **B**. Following stable expression of miR-125b, cell lysates were collected and analyzed for expression of HER2 as a known downstream target of this miR. As expected, there was a reduction in HER2 expression level when compared to vector control and parental cell lines. **C**. Cell proliferation rate was measured by manual counting of cells plated in triplicate for each time point indicated. Data shown are representative of three independent experiments and are shown as mean ± SEM.

**2.3. Confirming metastatic potential of MCF10 CA d1.α and KPL4 breast cancer cells.**

We stated in our original proposal that we would attempt to identify the molecular interplay between BRMS1, HER2 and miR-125b using two well-characterized metastatic breast cancer cell lines, MDA-231 and MDA-435. However, both of these cell lines are considered "normal" HER2 expressers, i.e. HER2 is not amplified. Therefore, we screened several other metastatic breast cancer cell lines and identified MCF10-CA-d1.α and KPL4 as two cell lines where HER2 protein expression level is elevated (data not shown). Specifically, KPL4 were reported to exhibit a 15-fold HER2 gene amplification (9), while MCF10-CA-d1.α exhibit HER2 protein level higher than that of MDA-231 or MDA-435 cells.

We then wanted to ensure that in our hands, these two reportedly metastatic (9-10) cell lines would produce metastases *in vivo*. Hence, we performed two pilot animal studies using these cell lines by injecting them intravenously (MCF10-CA-d1.α) or via intracardiac route (KPL4). As expected, both cell lines were highly metastatic (Figure 3). We are currently in the process of establishing variants of these two cell lines that stably express GFP-Luciferase fusion protein under control of the CMV promoter. Expression of GFP and Luciferase will enable us to better assess systemic disease upon injection of these cells into the animals, as well as better control of the experimental timeline. While our original proposal specified use of mKate2 instead of GFP, our current technical capabilities will preclude us from using a far-red fluorescent protein for *in vivo* or *ex vivo* imaging.

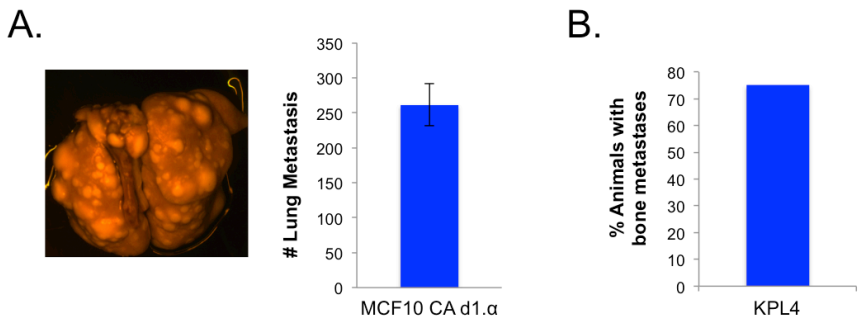


**Figure 3. MCF10 CA d1.α and KPL4 are metastatic *in vivo*. A**. MCF10 CA d1.α cells were injected intravenously and animals monitored for signs of lung metastases, such as dyspnea and wasting. All mice were sacrificed at week 7 post-injection, lungs fixed in Bowen's buffer and number of surface metastatic nodules counted under low magnification dissecting microscope. Data shown are one representative whole lung specimen with white metastatic colonies. Bar graph shows a mean number of nodules counted for all five mice in the study ± SEM. **B**. KPL4 cells were injected systemically via the left ventricle of the heart. Animals were monitored for signs of bone metastases, such as hind leg paralysis, hunched posture and wasting. All mice were sacrificed at different times upon detection of above symptoms. In total, ¾ animals developed presumed bone lesions, although histologic examination is yet to be completed.

**2.4. BRMS1 has no significant effect on expression of miR-125b and HER2.**

Based on our preliminary data from MDA-435 cells, we expected about 2-3 fold enhancement of miR-125b expression in BRMS1-expressing cells. Furthermore, we expected about 50% reduction in HER2 levels in BRMS1 expressing cells. As shown in Figure 4A, miR-125b levels were enhanced by BRMS1 expression in both MDA-231 and MDA-435 cell lines, although not to the degree expected. Moreover, there is a great variation in miR-125b expression level from one

experiment to the next, suggesting that expression of this microRNA may be relatively low in these cells, a possibility further supported by a high (between 32-34) cycle number when this microRNA is detected.

We then assessed level of HER2 expression by qPCR to gain insight into whether miR-125b may regulate HER2 at the level of transcription. As shown in Figure 4B (red bars), there was a much greater decrease in HER2 gene expression in MDA-435 cells as compared to MDA-231 cells. This decrease in HER2 expression also translated into a detectable decrease in HER2 protein level in these cells (Figure 4C). Conversely, in MDA-231 cells there was a much lower reduction in HER2 gene expression (blue bars, Figure 4B) and no detectable change in HER2 protein level (Figure 4C) between vector control and BRMS1-expressing cells.

Since it is thought that amplification of HER2 gene and/or elevation of its protein level may affect cell's behavior, we developed MCF10-CA-d1.α and KPL4 cells stably expressing BRMS1. As shown in Figure 4C, both cell lines have low endogenous BRMS1 expression. However, upon stable transfection of BRMS1 driven by CMV promoter (and tagged with Flag epitope at the N-terminus), no detectable change in HER2 protein level was detected.

From this set of experiments we therefore conclude that BRMS1 has only a marginal effect on miR-125b expression and no cell line independent effect on HER2 protein expression in these breast cancer cells.

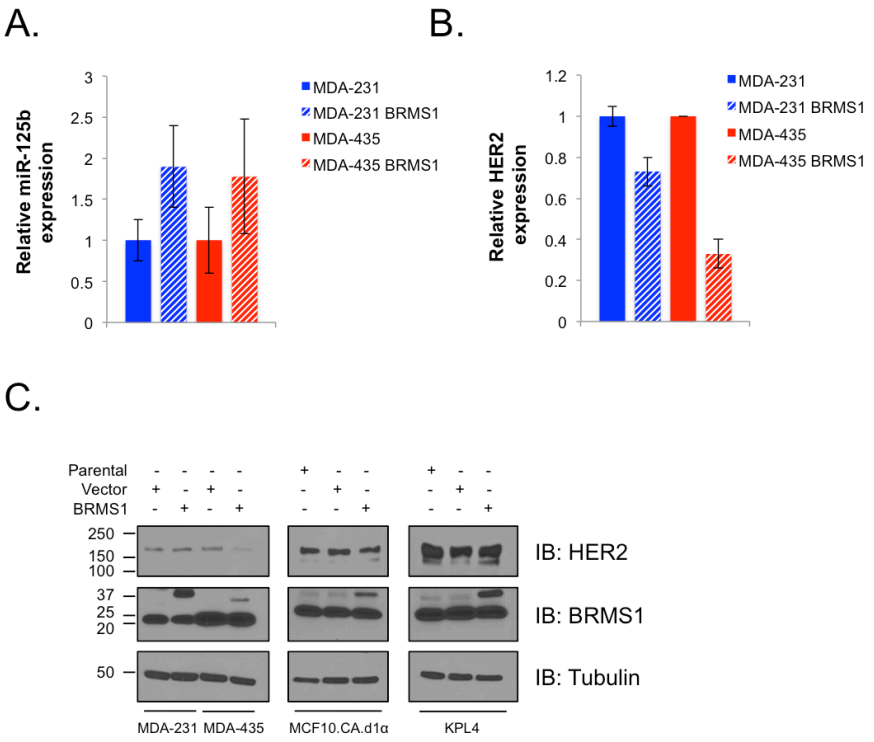


**Figure 4. BRMS1 has no significant effect on expression of miR-125b and HER2. A**. Total RNA was collected from cells using Qiazol reagent and expression of miR-125b assessed by qRCR. Data shown as mean relative expression of cells plated in triplicate ± SD. Data are representative of at least two independent experiments. **B**. Total RNA was collected from cells using Qiazol reagent and expression of HER2 assessed by qPCR. Data shown as mean relative expression of cells plated in triplicate ± SD. **C**. Total cell lysates were collected, resolved by SDS-PAGE and protein transferred onto PVDF membrane. Membranes were immunoblotted for HER2, BRMS1, and tubulin as a protein loading control.

While the work outlined in 2.4 is not a perfect match to the original SOW, additional cell lines were included because they exhibit HER2 gene amplification (KPL4) and high levels of HER2 protein (KPL4 and MCF10-CA-d1.α) and are representative of human HER2+ breast cancer classification, versus MDA-MB-231 and MDA-MB-435 cell lines that exhibit basal HER2 levels. We feel that by including these additional cell lines we enhanced relevance of our studies to the human disease. Moreover, including these additional cell lines were necessitated by our finding that BRMS1 only suppressed HER2 expression in MDA-MB-435, but not in MDA-MB-231, cell line. Consequently, we were able to show that out of four cell lines tested, HER2 expression was only suppressed in MDA-MB-435 cells. This finding lead us to question our original hypothesis that BRMS1 regulates expression of HER2.

PART II.

In the process of characterizing BRMS1-induced behavior that might account for enhanced anoikis and reduced survival during cell circulation *in vivo*, we observed that BRMS1 expression results in a delayed cell adhesion. To investigate this finding further we performed the following set of experiments and are currently in the process of finalizing data for publication (see Reportable Outcomes, manuscript in preparation).

Summary Review raised a concern that the original Progress Report we submitted outlined results of studies (such as adhesion and MET studies, below) not approved in the SOW. While we agree that these studies were not originally proposed, we feel that they are highly relevant. One of the points discussed in the original application is that BRMS1-expressing cells are less capable of reaching the bone through the circulatory system. Our original hypothesis was that downregulation of HER2 contributed to this “anoikis” phenotype. We further hypothesized that reduced TNF-α expression contributed to reduced growth of BRMS1-expressing cells in the bone. However, seeing that BRMS1 does not significantly affect HER2 or TNF-α expression, we were able to show that BRMS1-expressing cells exhibit retarded adhesion dynamics through delayed cytoskeletal rearrangement and activation of adhesion-associated signaling pathways. Moreover, we made an interesting observation that BRMS1 delays mesenchymal-to-epithelial transition, which may also contribute to BRMS1-associated anoikis.

**2.5. Expression of BRMS1 delays adhesion of breast cancer cells to matrix.**

Vector control and BRMS-expressing MDA-231 and MDA-435 breast cancer cells were plated on plates pre-coated with FBS to simulate conditions encountered by cells during *in vivo* dissemination. Time-lapse microscopy of live, unlabeled cells was utilized to assess adhesion kinetics. Essentially, we propose that a cell that dissociated from a primary tumor and entered circulation in order to metastasize must undergo a morphologic change from round (in circulation) to eventually fully attached and spread (Figure 5A). There are also a number of signaling changes associated with this morphologic transformation, starting with formation of focal adhesions and activation of integrins and culminating in transcriptional activation.

As shown in Figure 5B, BRMS1 expression greatly inhibited adhesion kinetics. Although if left undisturbed BRMS1-expressing cells eventually adhere, adhesion takes a much longer time.

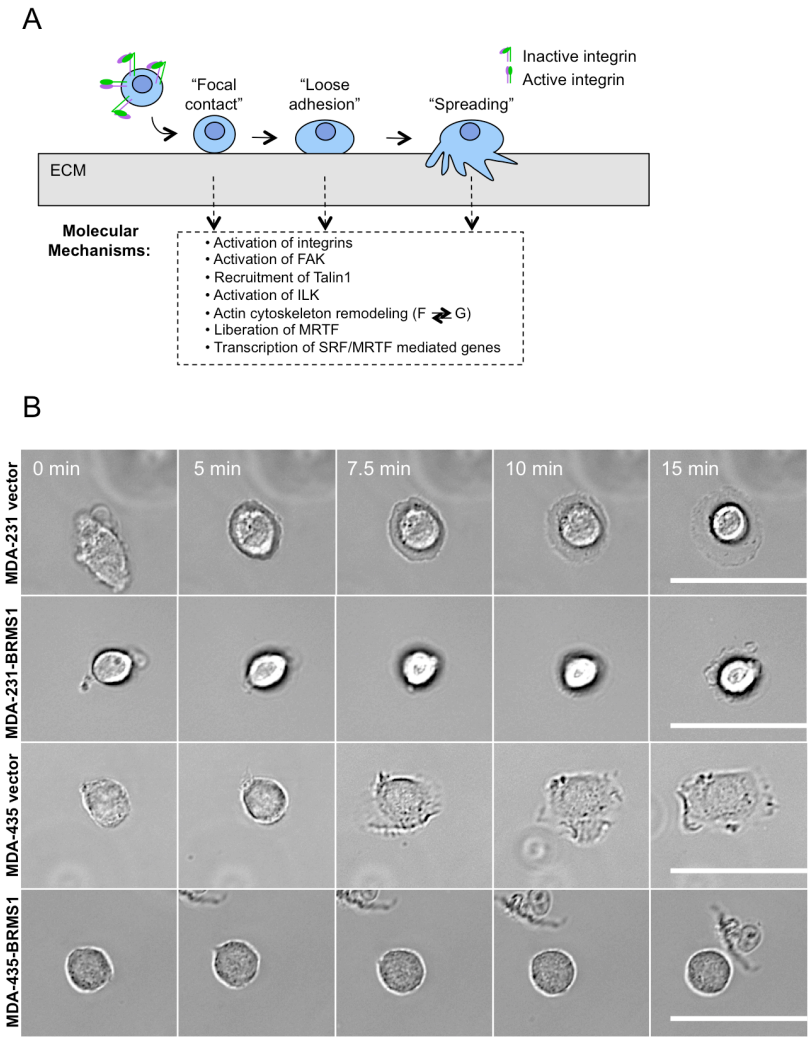


**Figure 5. BRMS1 delays adhesion of MDA-231 and MDA-435 breast cancer cells. A**. Model of adhesion examined. **B**. Vector control and BRMS1-expressing cells were plated on FBS-coated optical plates and imaged by time-lapse microscopy for one hour. Scale bar = 50μm.

**2.6. BRMS1 decreases focal adhesion number and inhibits its associated signaling.**

It has been previously shown that adhesion is mediated through assembly of and signaling through focal adhesions at the cell surface (11). We thus examined whether BRMS1 expression played a role in modulating expression or activity of major players in focal adhesion assembly: integrins, focal adhesion kinase (FAK), talin, or integrin linked kinase (ILK).

One of the first steps in formation of focal adhesions is the activation of integrins. Integrin β1 is the main binding partner for most α subunits. Therefore, examining activation status of this integrin can serve as a read-out for activation of many integrin heterodimers. As shown in Figure 6A, upon plating cell suspension onto FBS-coated plates and allowing cells to attach for 15 minutes, activated β1 integrin localizes to the outer-most plasma membrane (indicated by green spots, arrows). However, in cells stably expressing BRMS1 cell spreading is greatly reduced (Figure 5B) and localization of activated β1 integrin to focal adhesions is hampered (Figure 6A).

The next step in activation of focal adhesions is recruitment FAK and its phosphorylation by Src kinase. As shown in Figure 6B, cells expressing BRMS1 exhibit a dramatic decrease in cell spreading, evidenced by highly condensed cytoplasm and reduced actin cytoskeleton remodeling. Furthermore, localization of phosphorylated FAK (pFAK) to the plasma membrane is also greatly decreased (Figure 6B, arrows).

To determine key players in assembly and functionality of focal adhesions affected by BRMS1 expression, we preformed a time course experiment where cells in suspension were allowed to

attach to FBS-coated plates for times indicated, then lysed and examined by immunoblotting. As shown in Figure 6C, there is a significant decrease in levels of all components of the focal adhesion signaling complex examined: talin, pFAK, and ILK. Immunoblotting results were quantified and data shown in Figure 6D, 6E.

Overall, from these data we conclude that BRMS1 expression delays cell attachment to matrix by hampering integrin activation with consequent inhibition of focal adhesion signaling. We also show that this phenomenon is independent of type of matrix used (data not shown).

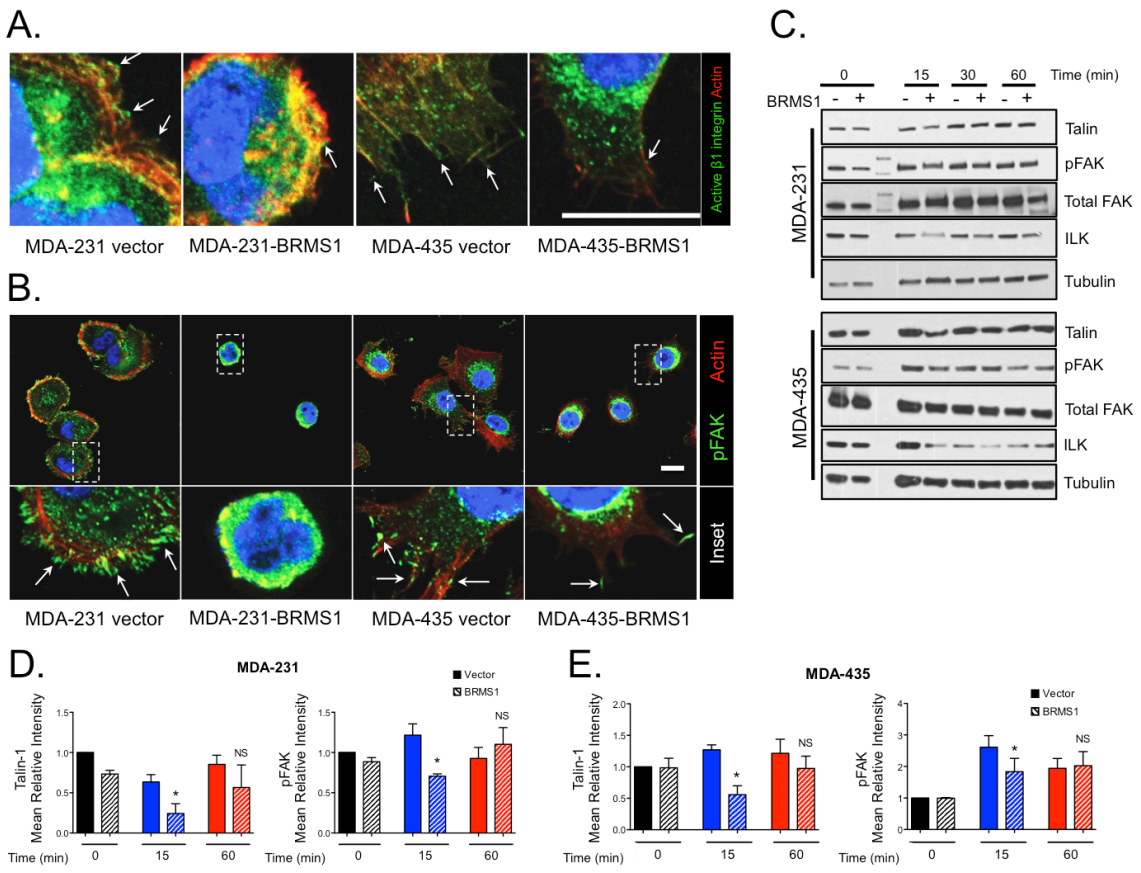


**Figure 6. Expression of BRMS1 in inhibits formation of focal adhesion complexes. A**. Vector control and BRMS1-expressing breast cancer cells were plated onto chamber slides pre-coated with FBS and allowed to adhere for 15 minutes. Fixed cells were immunostained for actin (red) and activated β1 integrin (green). Arrows point to areas staining for activated β1 integrin and localized to the outer-most plasma membrane. Scale bar = 20μm. **B**. Vector control and BRMS1-expressing cells were plated onto chamber slides pre-coated with FBS and allowed to adhere for 30 minutes. Fixed cells were stained for actin (red) and pFAK (green). Arrows point to focal adhesions, as indicated by green staining. Scale bar = 20μm. **C**. Vector and BRMS1-expressing cells were plated onto plates pre-coated with FBS and lysed at time points indicated. Cell lysates were assayed by western blotting for levels of focal adhesion markers. Expression of Talin and pFAK from three independent experiments were enumerated using ImageJ and data are shown in **D** and **E**. Statistical analysis was performed using T-test. *p<0.05

## 2.7. BRMS1 may play a role in mesenchymal-to-epithelial transition (MET).

It is currently thought that cells that metastasize must change their physical appearance during their journey from primary tumor to a distant site. Specifically, it is believed that mesenchymal cells possess qualities that yield in their escape from the primary tumor, while cells that acquire an epithelial phenotype are better at colonizing secondary sites (12). This EMT/MET switch manifests itself, in part, through modulation in expression of epithelial (E-cadherin) and mesenchymal (ZEB1, Snail) markers. Because we have previously shown that BRMS1

expressing cells remain as single cells at secondary sites (13-14) and our current data point to BRMS1-induced inability of cells to properly respond to their microenvironment, we hypothesized that BRMS1 may inhibit MET.

We first examined expression of E-cadherin, ZEB1, and Snail in vector and BRMS1-expressing breast cancer cells grown in normal culture. As shown in Figure 7A, there was little difference in expression of ZEB1 and Snail, while E-cadherin was not detected in these cells (data not shown). These data indicated that under normal conditions, both of these cell lines exhibit mesenchymal characteristics. We then reasoned that our adhesion assay may be useful in assessing forced morphologic change a cell must undergo upon arriving at the secondary site (MET). As shown in Figure 7B, upon plating cell suspension onto FBS-coated plates, expression of ZEB1 and Snail changes. Interestingly, expression of Snail is reduced in BRMS1-expressing cells at an earlier time point (15 minutes) while expression of ZEB1 is reduced after 1 hour post-plating. These data might indicate sequential regulation of these mesenchymal markers. However, data also suggest that BRMS1 may delay MET when cells reach secondary sites, which may also lead to their reduced survival in circulation *in vivo*.

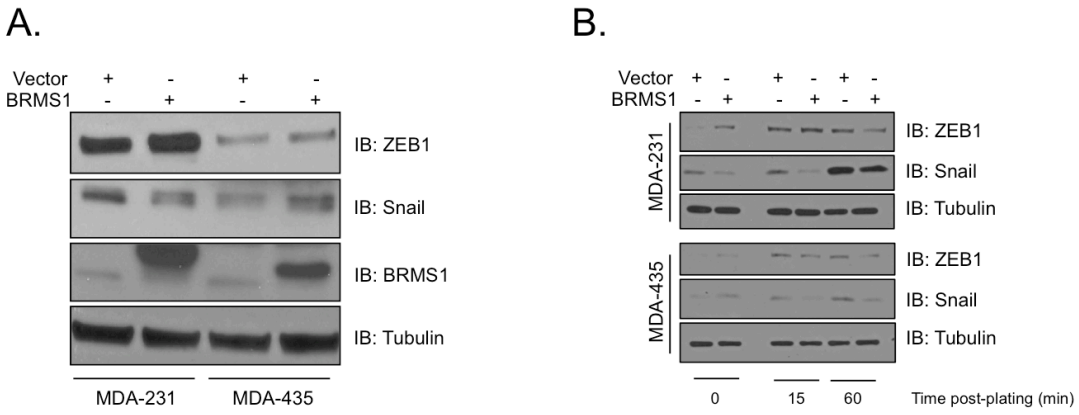


**Figure 7. BRMS1 may inhibit MET. A**. Vector and BRMS1-expressing cell lysates were collected from cells growing in normal culture and analyzed for expression of epithelial and mesenchymal markers by immunoblotting. **B**. Vector and BRMS1-expressing cells were plated onto FBS-coated plates and lysates collected at times indicated. Lysates were analyzed for expression of mesenchymal markers by immunoblotting.

### 2.8. Concerns about original collaborations

During the review of the original progress report, a concern was raised regarding our collaboration with investigators at UAB. As you may already know, Dr. Danny Welch had since moved from UAB to the Kansas University Medical Center. We continue our collaboration with Dr. Welch and his former postdoc, Dr. Douglas Hurst, who is currently an Assistant Professor in the Department of Pathology at UAB. However, we could not continue our collaboration with Dr. Andra Frost, a clinical pathologist from UAB, due to Dr. Frost's departmental service restrictions. Consequently, Dr. Weiya Xia, a clinical pathologist in Dr. Hung's lab at MDACC, has been working with us on all human tissue-associated immunohistochemistry. Furthermore, our collaboration with Drs. Zayzafoon and Feng from UAB is also questionable, mainly because our original hypothesis may not be fully correct. However, should we need their expertise, they will be willing to uphold their original agreement of collaboration.

## 3. Future work

3.1. Because BRMS1 has a pronounced effect on cells' cytoskeleton (both actin and microtubules), we will utilize our newly established cells expressing Flag-tagged BRMS1 to determine whether BRMS1 directly interacts with members of the cytoskeleton. We believe that BRMS1 may have a yet unknown role restricted to cytosole based on two key pieces of evidence: i) BRMS1 is detected in cytosole of most breast cancer tumor biopsies, yet it is presumed role as part of Sin3A complex is restricted to the nucleus and ii) BRMS1 has been shown to interact with HDAC6, a cytosolic protein that regulates stability of microtubules. We will isolate BRMS1-associated proteins by performing immunoprecipitation (i.p.) and subject utilize i.p. products to mass spectrometry to identify proteins that differentially bind to BRMS1 in the nucleus vs. cytosole. If BRMS1 and HDAC6 can interact and/or regulate one another's function, we will also utilize inhibitors specific for HDAC6 to determine whether they could enhance suppression of metastasis in cells with normal BRMS1 expression.

3.2. We recently observed that BRMS1 may have an effect on MET. We will continue to work on identifying a potential molecular mechanism underlying this phenomenon. Specifically, we will try to delineate why Snail is reduced earlier and ZEB1 is reduced later in the process of cell attachment/adhesion and how BRMS1 may regulate them. Presumably, BRMS1 could be involved in regulating the transcription of these two genes through its activity within Sin3A complex. Moreover, we recently observed that BRMS1 is involved in regulation of levels of cytoskeleton-dependent serum response factor (SRF) transcription factor. Many genes that play a role in adhesion, migration, and proliferation are under the control of SRF. Interestingly, one recent article proposed that Snail expression is dependent on presence of sera in the media, raising a question whether BRMS1 regulates Snail through its regulation of SRF.

3.3. To further examine the role of BRMS1 in MET and regulation of adhesion/colonization of secondary sites, we would like to also propose some *in vivo* studies. We will utilize cells with or without BRMS1 expression that are tagged with eGFP-Luciferase fusion protein. Following either spontaneous metastases of cells grown in the mammary fat pad or after intravenous injection, we will isolate lungs. Some lungs will be stained for betaglycosidase, a marker of senescence, to determine if BRMS1-expressing cells are unable to colonize secondary sites because they are senescent. Cells from other sets of lungs will be isolated and screened through non-biased approaches (gene array, Chip array to examine epigenomic status of cells) for markers of MET. We may also perform this experiment in a time-course setting to determine whether time-specific regulation of Snail and ZEB1 are relevant to behavior of cells *in vivo*.

## 4. Key Research Accomplishments

4.1. Confirmed inverse correlation between BRMS1 and HER2 in human breast cancer biopsy samples.
4.2. Confirmed that miR-125b inhibits HER2 expression.
4.3. Established that BRMS1 has only a marginal effect on expression of HER2 and miR-125b.
4.4. Determined that neither MDA-MB-231 nor MDA-MB-435 express TNF-α.
4.5. Confirmed metastatic capability of MCF10-CA-d1.α and KPL4 *in vivo.*
4.6. Cloned Flag tagged (N-terminus) BRMS1 into lentiviral plasmid to be driven by CMV promoter. Developed another lentiviral plasmid containing fusion eGFP-Luciferase protein.
4.7. Established two epithelial breast cancer cell lines with elevated HER2 expression (MCF10 CA d1.α and KPL4) to stably express BRMS.
4.8. Identified a possible mechanism underlying BRMS1-induced anoikis.
4.9. Identified BRMS1 as a potential mediator of MET.

## 5. Reportable outcomes

5.1. Khotskaya YB, Beck BH, Hurst DR, Hung MC, and Welch DR. (2011) Breast cancer metastasis suppressor 1 (BRMS1) suppresses attachment and spreading of breast cancer cells on 2D and 3D extracellular matrix components by altering focal adhesion-associated signaling. AACR Annual Meeting, Orlando, FL

5.2. Khotskaya YB, Beck BH, Hurst DR, Hung MC, and Welch DR. (2011) BRMS1 inhibits breast cancer metastases by inhibiting cells' ability to interact with collagen I. DOD Era of Hope, Orlando, FL

## 6. Conclusion

6.1. BRMS1 has no significant effect regulating miR-125b and HER2 expression. It is likely that inverse correlation between HER2 and BRMS1 observed in breast cancer tumor samples arises from other mechanisms.

6.2. BRMS1 regulates cell adhesion through inhibition of focal adhesions.

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# Breast cancer metastasis suppressor 1 (BRMS1) suppresses attachment and spreading of breast cancer cells on 2D and 3D extracellular matrix components by altering focal adhesion-associated signaling.

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Metastatic dissemination of cancer cells from primary tumor to secondary sites is a multi-step process that depends heavily on the ability of cancer cells to respond to the microenvironmental cues, such as changes in composition of surrounding extracellular matrix (ECM), by adapting their adhesion abilities and undergoing cytoskeletal rearrangement. Many of these interactions between cancer cells and ECM are mediated by signaling cascades initiated at the cell surface through activation of integrins and growth factor receptors. BRMS1, or Breast cancer Metastasis Suppressor 1, belongs to a family of metastasis suppressor genes and has been shown to affect several steps of the metastatic cascade. BRMS1-expressing cells shed by the primary tumor can enter the circulation and reach secondary sites, where they remain largely as single cells. Moreover, ectopic expression of BRMS1 results in a significant decrease in survival of tumor cells within blood stream, increased susceptibility to anoikis and inability to form colonies at secondary organ sites, all events that could be attributed to failure of BRMS1 expressing cells to activate integrins and strongly adhere to ECM components. Here, we show that BRMS1 expression in MDA-MB-231 and MDA-MB-435 cells did not significantly alter expression levels of the integrin monomers tested. However, after short-term (15-30 minutes) plating of cells onto mixed ECM or individual ECM components (collagen I, collagen IV, or fibronectin) under 2D conditions, BRMS1-expressing cells exhibited reduced activation of β1 integrin, focal adhesion kinase (FAK), and scaffolding protein Talin1, as well as a decrease in their localization to focal adhesions. Furthermore, short-term plating of BRMS1-expressing cells on collagens I or IV or fibronectin resulted in marked inhibition of cytoskeletal rearrangement and failure to form cellular adhesion projections, as compared to cells vector-transfected cells. In addition, under 3D conditions, BRMS1-expressing cells remained rounded and failed to reorganize their cytoskeleton even after 24-hour stimulation with serum. Taken together, we believe that these findings demonstrate that BRMS1-expressing breast cancer cells are inherently unable to respond to microenvironment changes, which may explain why they exhibit reduced survival in circulation, increased susceptibility to anoikis, and decreased colonization of secondary sites.

YBK was supported in part by NIH T32 (AR047512-09) and DOD CDMRP BCRP Postdoctoral Fellowship (BC096855)

**Appendix 2 (Copy of Abstract presented at the Era of Hope Meeting in Orlando, FL)**

# BRMS1 inhibits breast cancer metastases by inhibiting cells' ability to interact with collagen I

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Breast cancer metastasis is a complex, multi-step process that relies on various cues from the surrounding microenvironment including matrix proteins, growth factors, stromal and immune cells. Extracellular matrix (ECM) proteins are diverse and include collagens, fibronectin and laminin. Type I collagen is the most abundant collagen in the human body, playing a major role in maintenance of physiologic architecture of blood vessels, skin, bone and lungs. Pathologically, collagen I is the major component of fibrotic tissues and has been implicated in stimulating re-growth of dormant cancer cells into metastatic lesions. BRMS1, or Breast cancer Metastasis Suppressor 1, belongs to a family of metastasis suppressor genes and has been shown to affect several steps of the metastatic cascade. BRMS1-expressing cells shed by the primary tumor can enter the circulation and reach secondary sites, where they remain largely as single cells. Moreover, ectopic expression of BRMS1 results in a significant decrease in survival of tumor cells within the blood stream, increased susceptibility to anoikis and inability to form colonies at secondary organ sites, all events that could be attributed to failure of BRMS1 expressing cells to appropriately interact with ECM components. Here, we show that collagen I induces both short-term and long-term effects on BRMS1-expressing MDA-MB-231 and MDA-MB-435 breast cancer cells. Specifically, BRMS1-expressing cells plated on collagen I short-term exhibited reduced activation of focal adhesion kinase (FAK) and scaffolding protein Talin1, as well as a decrease in their localization to focal adhesions. Furthermore, short-term plating of BRMS1-expressing cells on collagens I resulted in marked inhibition of cytoskeletal rearrangement and failure to form cellular adhesion projections, as compared to cells vector-transfected cells. In addition, under 3D conditions, BRMS1-expressing cells remained rounded and failed to reorganize their cytoskeleton even after 48-hour stimulation with serum. Long-term, BRMS1-expressing breast cancer cells plated in 3D collagen I for 8 days formed smaller and less invasive colonies, although the overall number of colonies formed was similar to that of the vector control group. Smaller colony size was not due to increase in apoptosis, indicating a possible initiation of cellular dormancy. Moreover, following long-term culture in collagen I, BRMS1-expressing cells exhibit reduced levels of myocardin-related transcription factor A (MRTF-A) corresponding with reduction in RhoA, further indicating alterations in actin cytodynamics. Taken together, these data may explain initiation of BRMS1-associated anoikis during cancer cell trafficking from primary tumor to a secondary site. These data also suggest that failure of BRMS1-expressing cells to grow extensively at the secondary site may be due to initiation of dormancy caused by loss of interaction with collagen I. Further studies will begin to elucidate whether this property of BRMS1 could be exploited for therapeutic purposes.

YBK was supported in part by NIH T32 (AR047512-09) and DOD CDMRP BCRP Postdoctoral Fellowship (BC096855)